ATP Augments Peptide Release from Rat Sensory Neurons in Culture through Activation of P2Y Receptors

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ABSTRACT

ATP has recently emerged as an important proinflammatory mediator that has direct excitatory actions on sensory neurons through activation of ion channel-coupled P2X receptors. The purpose of the current work is to assess whether ATP alters the release of neuropeptides from sensory neurons and the receptors mediating this putative action. Exposure of embryonic sensory neurons in culture to concentrations of ATP up to 300 μM did not increase the release of immunoreactive substance P or calcitonin gene-related peptide from sensory neurons. However, pre-exposing sensory neurons to 0.1 to 100 μM ATP prior to and throughout administration of 30 nM capsaicin resulted in a significant augmentation of release evoked by the vanilloid. This sensitizing action of ATP is blocked by suramin but not a significant augmentation of release evoked by the vanilloid.

ABBREVIATIONS: α,β-methyleneadenosine 5′-diphosphate; iCGRP, immunoreactive calcitonin gene-related peptide; iSP, immunoreactive substance P; DRG, dorsal root ganglion; PPADS, pyridoxal phosphate-6-azobenzene-2,4-disulfonic acid; 2-MeSATP, 2-(methylthio) adenosine 5′-triphosphate; β,γ-meATP, β,γ-methyleneadenosine 5′-diphosphate; 2-CiATP, 2-chloroadenosine triphosphate; PKC, protein kinase C.
It is clear that ATP evokes excitatory responses in sensory neurons, but controversy remains as to whether the nociceptive actions of ATP are caused by direct excitation of sensory neurons or through the ability of ATP to augment the excitability to other stimuli. In vivo, the P2X receptor agonist, α,β-meATP, is unable to excite corneal nociceptors (Dowd et al., 1997) or tooth pulp afferents in cats (Matthews et al., 1997), whereas intra-arterial injections of ATP or α,β-meATP into the knee joint in rats evokes a rapid and short-lasting excitation of C and Aδ fibers innervating knee joints (Dowd et al., 1998). Transgenic P2X2-/-null mice show normal response to acute mechanical and thermal stimuli (Cockayne et al., 2000; Souslova et al., 2000), suggesting that P2X2 is not essential in acute nociception. In isolated sensory neurons, Sanada et al. (2002) showed that exposure to high concentrations of ATP or UTP increased intracellular calcium and that UTP (100 μM) directly induced release of immunoreactive calcitonin gene-related peptide (iCGRP) from rat sensory neurons. In contrast, Zimmermann et al. (2002) demonstrated that 100 μM ATP has little, if any, direct excitatory effects on the release of iCGRP from the nerve terminals in isolated rat dura mater; rather, it augments proton-induced release of iCGRP. Whether this action of ATP was due to a direct effect on sensory neurons was not determined in this study.

Given the effects of ATP on sensory neurons through P2X and P2Y receptors, we sought to determine whether ATP, P2X agonists, and P2Y agonists could increase the release of and/or augment the capsaicin-stimulated release of immunoreactive substance P (iSP) and iCGRP from sensory neurons grown in culture. We also ascertained which subtypes of the P2 purinergic receptors mediate the effect of ATP, and whether these putative subtypes are expressed in DRG sensory neurons. Our results indicate that ATP augments the release of iSP and iCGRP evoked by capsaicin in rat sensory neurons in culture through activation of P2Y receptors, but does not alter basal peptide release when administered alone. Portions of this work appeared previously in abstract form (Wu et al., 1997).

**Materials and Methods**

**Materials.** Timed-pregnant Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA), and nerve growth factor was obtained from Harlan Bioproducts for Science (Indianapolis, IN). Peptides were obtained from Peninsula Laboratories (Belmont, CA). Suramin, pyridoxal phosphate-6-azobenzene-2,4-disulfonic acid (PPADS), 2-(methylthio)adenosine 5'-triphosphate (2-MeSATP), α,β-methyleneadenosine 5'-diphosphate (α,β-meATP), β,γ-methyleneadenosine 5'-diphosphate (β,γ-meATP), 2-chloroadenosine triphosphate (2-ClATP), uridine 5'-triphosphate (UTP), adenosine 5'-triphosphate (ATP), capsaicin, and 1-methyl-2-pyrrrolidinone were obtained from Sigma-Aldrich (St. Louis, MO). Bisindolylmaleimide I was purchased from Calbiochem (San Diego, CA). Capsaicin was initially dissolved in 1-methyl-2-pyrrrolidinone (Aldrich Chemical Co., Milwaukee, WI) and then diluted to appropriate concentrations with HEPES buffer. This vehicle did not alter the release of either peptide at the concentrations used. Polyclonal antibodies against P2Y1, P2Y5, and P2Y4 and their corresponding control antigens were obtained from Chemicon International (Temecula, CA).

**Isolation and Culture of Embryonic Rat Sensory Neurons.** Cultures of sensory neurons were prepared using a modification of our previously described protocol (Vasko et al., 1994). Briefly, cells were dissociated from the dorsal root ganglia of 15- to 17-day rat embryos using trypsin and mechanical dissociation. Approximately 150,000 cells were plated into poly-D-lysine-coated wells of 24-well Falcon culture dish. The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 50 μg/ml penicillin and streptomycin, 10% (v/v) heat-inactivated fetal bovine serum, 50 μM 5-fluro-2'-deoxyuridine, and 250 ng/ml nerve growth factor. Cultures were maintained at 37°C under a 5% CO2 atmosphere. The medium was replaced every 2 to 3 days.

**Release of iSP and iCGRP from Sensory Neurons.** After 9 to 12 days in culture, release of iSP and iCGRP from cultured sensory neurons was determined as previously described (Vasko et al., 1994). We chose to perform experiments at this time to allow sufficient neuropeptides to accumulate so that we could reproducibly measure resting levels of release. Cells were incubated for successive 10-min intervals in 0.4 ml of HEPES buffer consisting of 25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM D-glucose, 1 μM phosphoramidon, and 0.1% (w/v) bovine serum albumin (pH 7.4), and maintained at 37°C.

Basal neuropeptide release was determined by exposing cells to HEPES buffer alone for 10 min, whereas the direct effect of purinergic agonists or antagonists on release was determined by exposing cells for the next 10 min to drugs. This was followed by incubating the cells for 10 min in buffer containing 30 nM capsaicin in the absence or presence of purinergic agonists and antagonists. Subsequently, cells were incubated for 10 min with HEPES buffer alone to show a return to baseline. After each 10-min incubation, the buffer was removed, aliquoted, and assayed for iSP and iCGRP by radioimmunoassay as previously described (Vasko et al., 1994).

**Immunoblotting.** Cultured sensory neurons were washed in phosphate-buffered saline, scraped, and pelleted at 10,000 g for 2 min. Cells were resuspended in radioimmunoprecipitation assay buffer containing 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride, 3% aprotinin, and 1 mM sodium orthovanadate, and then disrupted by sonication (two 15-s pulses). Protein concentration in the lysate was determined by using a Bradford protein assay. Cell lysates (40 μg/lane) were separated on 10% sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked by incubation in 5% Carnation instant milk in Tris-buffered saline for 1 h. Subsequently, the membrane was incubated with a primary antibody for 1 h. The membrane was incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. Immunoreactive bands were developed by an ECL kit (PerkinElmer Life Sciences, Boston, MA), and visualized by exposure to film.

**Statistical Analysis.** Data are presented as mean ± S.E.M. with n being the total number of wells used. ANOVA was used to compare release stimulated by capsaicin alone with release evoked by capsaicin and various purinergic agents. If this test indicated that a difference existed, post hoc Bonferroni tests were performed. The significance level for all tests was set at P < 0.05. In some instances, the capsaicin-evoked release varied between experiments, and thus, the data were normalized to percentage of control capsaicin-evoked release.

**Results**

**Effects of ATP on Peptide Release from Cultures of Rat Sensory Neurons.** To determine whether ATP directly alters peptide release from sensory neurons, neuronal cultures were exposed to different concentrations of ATP for 10
As shown in Fig. 1 (dashed lines), at concentrations ranging from 0.1 μM to 300 μM, ATP did not increase the release of iSP and iCGRP when compared to basal neuropeptide release in the presence of HEPES buffer alone.

To assess whether ATP could augment release evoked by capsaicin, cultures were exposed to 0.1, 1.0, 10, 100, or 300 μM ATP for 10 min prior to and throughout treatment with 30 nM capsaicin. Exposing sensory neurons to 30 nM capsaicin resulted in a significant increase in the release of iSP and iCGRP from basal levels of 10 ± 0.4 and 31 ± 2 fmol/well/10 min to 108 ± 10 and 487 ± 56 fmol/well/10 min, respectively. When cells were exposed to ATP for 10 min prior to and throughout capsaicin treatment, all concentrations of ATP that were utilized except 300 μM resulted in an augmentation of capsaicin-evoked release of both iSP and iCGRP. For example, 1.0 μM ATP resulted in an approximately 68% increase in capsaicin-evoked release of iSP and an approximately 84% increase in iCGRP release above that observed with capsaicin alone. When the concentration of ATP was increased to 10 μM, capsaicin-evoked release of iSP release was increased from 117 ± 16 to 268 ± 39 fmol/well/10 min, whereas capsaicin-simulated release of iCGRP was elevated by ATP from 535 ± 74 to 1174 ± 86 fmol/well/10 min. These results suggest that although ATP does not increase release directly, it can sensitize sensory neurons as indicated by its ability to augment capsaicin-evoked release.

Effects of Purinoreceptor Antagonists on ATP-Induced Enhancement of Peptide Release from Cultures of Rat Sensory Neurons. In an attempt to define the receptor subtypes mediating the sensitizing actions of ATP on sensory neurons, we examined the effects of two purinoreceptor antagonists on ATP-induced augmentation of capsaicin-evoked neuropeptide release. For these studies, cells were exposed to various antagonists 10 min prior to and throughout treatment with 10 μM ATP and 30 nM capsaicin. We chose to use 10 μM ATP because this concentration produced the maximal effect on capsaicin-induced peptide release. Initial studies were performed to assess whether suramin, a P2 antagonist, could attenuate the effects of ATP (Fig. 2), to distinguish between P2 receptors and P1 (adenosine A1 and A2) receptors. As with previous results, when sensory neurons were pretreated with 10 μM ATP, capsaicin-evoked release of iSP and iCGRP was elevated by approximately 1.4- to 1.5-fold compared to release with capsaicin alone (Fig. 2). Capsaicin-induced release of iSP was increased from 160 ± 3 to 241 ± 11 fmol/well/10 min, whereas stimulated release of iCGRP was elevated by ATP from 1209 ± 48 fmol/well/10 min to 1717 ± 91 fmol/well/10 min. When additional cultures from the same harvests were exposed to 30 μM suramin 10 min prior to and throughout treatment with ATP, the P2 antagonist significantly attenuated the actions of ATP (Fig. 2). Capsaicin-evoked release of iSP and iCGRP in the presence of suramin and ATP was reduced to 164 ± 4 fmol/well/10 min and 1293 ± 39 fmol/well/10 min, respectively. As a control, we also assessed whether suramin at the concentration used would alter capsaicin-evoked release of peptides. This nonselective P2 receptor antagonist did not have any significant effect on capsaicin-evoked release in the absence of ATP. Capsaicin-evoked release in control cells was 45 ± 3 and 304 ± 14 fmol/well/10 min for iSP and iCGRP, respectively, and 46 ± 3 and 338 ± 8 fmol/well/10 min in the presence of suramin alone. Together, these results suggest that the ATP-induced augmentation of peptide release is mediated by P2 purinoreceptors.

To examine whether the effects of ATP are mediated by P2X or P2Y receptors, further studies were performed using the relatively selective P2X antagonist, PPADS. As can be seen in Fig. 3, exposing neuronal cultures to 30 nM capsaicin and 10 μM ATP significantly increased capsaicin-evoked release of iSP by 1.4-fold and iCGRP by 1.6-fold. Pre-exposure to 10 μM PPADS did not attenuate the ATP-induced augmentation of capsaicin-evoked release of iSP and iCGRP, which are 1.6- and 1.8-fold higher than capsaicin-evoked release in control cells, respectively. This concentration of PPADS likely blocked a majority of P2X receptors since the IC50 for this compound at these receptors is 1 to 3 μM (Ralevic and Burnstock, 1998).
Effects of P2 Receptor Agonists on Peptide Release.

Release studies in the presence of purinoreceptor antagonists suggest that the ATP-induced increases in peptide release are mediated by P2Y receptors. To confirm this and further characterize the receptor subtype mediating sensitization, release studies were performed using P2X and P2Y receptor agonists. Because the relative selectivity of purinoreceptor agonists is limited, we sought to examine the effects of a number of agents and compiled the results.

Figure 4 shows summary data from a number of experiments using ATP receptor agonists to augment capsaicin-evoked release. In these studies, cells in culture were exposed to agonists at a concentration of 10 μM for 10 min prior to and throughout capsaicin treatment. None of the agonists tested had any effect on the basal release of either iSP or iCGRP (data not shown). Treating sensory neurons with putative P2X agonists, 2-MeSATP, α,β-meATP, and β,γ-meATP (North and Surprenant 2000), had mixed effects on the capsaicin-evoked release of iSP and iCGRP. As can be seen in Fig. 4, 10 μM 2-MeSATP did not have any significant effect on peptide release, whereas α,β-meATP, another P2X agonist, resulted in a small, but significant decrease in capsaicin-induced release of iSP (18% for iSP and 26% for iCGRP). In contrast, β,γ-meATP enhanced the capsaicin-induced release of iSP and iCGRP by approximately 1.7- and 1.6-fold, respectively. These results suggest that the sensitizing actions of ATP on evoked peptide release are not mediated by P2X receptors, since these agonists did not have a consistent effect on peptide release.

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We also examined the effects of two putative P2Y agonists, 2-ClATP and UTP, on peptide release. Exposing sensory neurons to a 10 μM concentration of either drug resulted in a significant increase in the capsaicin-evoked release of iSP and iCGRP. The increase in release was similar in magni-
tude to that caused by ATP, roughly 1.3- to 1.5-fold. These results, together with the data from experiments using purinoreceptor antagonists, are consistent with the notion that P2Y receptors mediate ATP enhancement of capsaicin-stimulated neuropeptide release from sensory neurons.

**P2Y2 Receptors Are Expressed by DRG Sensory Neurons.** Five subtypes of P2Y receptors have been identified in mammalian cells: P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors (von Kugelgen and Wetter, 2000). To confirm the existence of P2Y receptors on embryonic sensory neurons, we performed Western blots using P2Y1, P2Y2, and P2Y4 receptor antibodies with proteins extracted from neuronal cultures. Using anti-P2Y2 antibody, we detected an immunoreactive band at the appropriate molecular weight of P2Y2 receptor (Fig. 5A). Furthermore, preincubating the anti-P2Y2 antibody with P2Y2 control antigen eliminated the corresponding immunoreactive band (Fig. 5B). We did not observe bands corresponding to P2Y1 or P2Y4 receptor-like immunoreactivity in protein extracts from neuronal cultures. Using anti-P2Y2 antibody, we detected an immunoreactive band at the appropriate molecular weight of P2Y2 receptor (Fig. 5A). Furthermore, preincubating the anti-P2Y2 antibody with P2Y2 control antigen eliminated the corresponding immunoreactive band (Fig. 5B). We did not observe bands corresponding to P2Y1 or P2Y4 receptor-like immunoreactivity in protein extracts from neuronal cultures (Fig. 5A).

To confirm, however, that our immunoblotting technique could detect P2Y1 and P2Y4 receptor antibodies with proteins extracted from neuronal cultures (Fig. 5A). To confirm, however, that our immunoblotting technique could detect P2Y1 and P2Y4 receptor antibodies with proteins extracted from neuronal cultures (Fig. 5A). To confirm, however, that our immunoblotting technique could detect P2Y1 and P2Y4 receptor antibodies with proteins extracted from neuronal cultures (Fig. 5A).

**Effect of Bisindolylmaleimide I on ATP-Induced Sensitization of Sensory Neurons.** Because P2Y receptors activate PKC-dependent signal transduction pathways in other cell systems (von Kugelgen and Wetter, 2000), we examined whether the ATP-mediated sensitization of sensory neurons is inhibited by bisindolylmaleimide I, a PKC inhibitor. As shown in Fig. 6, when cultures of sensory neurons were exposed to 100 nM bisindolylmaleimide I throughout treatment with ATP, the PKC inhibitor significantly attenuated the actions of ATP. Exposing cultured sensory neurons to 30 nM capsaicin resulted in a significant increase in the release of iCGRP from a basal value of 27 ± 7 pmol/well/10 min to 194 ± 11 pmol/well/10 min. Pretreatment with 10 μM ATP significantly enhanced the capsaicin-evoked release by 1.5-fold to a level of 293 ± 8 pmol/well/10 min. When cells were treated with 100 nM bisindolylmaleimide I for 10 min prior to and throughout the exposure to capsaicin, the ATP augmentation of capsaicin-evoked iCGRP release was abolished (200 ± 8 pmol/well/10 min). The PKC inhibitor at this concentration did not alter the resting release of iCGRP, nor
did it significantly reduce the capsaicin-evoked release in the absence of ATP (right panel, Fig. 6).

Discussion

There are two major findings of the present study. First, neither ATP at concentrations from 0.1 to 300 \( \mu M \) nor P2 agonists at 10 \( \mu M \) increase the resting release of iSP or iCGRP from isolated sensory neurons. Rather, at these concentrations, ATP and select P2 agonists significantly augmented the peptide release evoked by capsaicin, showing that a major effect of activating purinoreceptors is to alter the sensitivity of sensory neurons. Second, the agonist and antagonist profile for producing or blocking sensitization strongly supports the notion that the actions of ATP are mediated by activation of P2Y rather than P2X receptors. We chose to examine the effects of ATP on isolated embryonic sensory neurons since the use of this preparation minimizes the likelihood that ATP activates other cells, inadvertently causing the release of other inflammatory mediators. Indeed, the use of in situ or in vitro methods to measure the effects of inflammatory mediators on activation of sensory neurons may be confounded by the fact that the exogenously administered ATP could cause release of endogenous substances that, in turn, alter the excitability of sensory neurons. Furthermore, we have extensively characterized this preparation to discern whether inflammatory mediators directly stimulate release or augment release evoked by other stimuli (Hingtgen and Vasko, 1994; Vasko et al., 1994). Based on our results, ATP belongs to the latter group because it enhances capsaicin-evoked peptide release without directly inducing any release.

It is well established that exposing sensory neurons to ATP results in activation of an inward current through actions on P2X\(_2\) and/or P2X\(_3\) receptors (Chen et al., 1995; Lewis et al., 1995; Gu and MacDermott, 1997). This ability to excite sensory neurons suggests that ATP should increase the release of transmitters from sensory neurons, yet our results, using peptide release from isolated sensory neurons as an endpoint, do not support this notion. One possible explanation for this inconsistency is that the effects of ATP at P2X receptor desensitize quickly and thus do not provide sufficient stimulus to evoke detectable release of neuropeptides. Multiple subtypes of P2X receptors rarely colocalize in DRG sensory neurons (Barden and Bennett, 2000), and small-diameter, capsaicin-sensitive sensory neurons mainly express homomeric P2X\(_3\) receptors (Ueno et al., 1999). These P2X\(_3\) receptors, unlike the P2X\(_2\)/P2X\(_3\) heteromeric receptors in medium-sized, capsaicin-insensitive sensory neurons, desensitize very quickly (<100 ms) and recover from desensitization very slowly (>20 min) (Lewis et al., 1995). Thus, the kinetics of P2X\(_3\) receptor make it unlikely that its activation can sensitize peptidergic neurons. Moreover, P2X\(_3\) null mice showed normal responses to noxious mechanical and thermal stimuli; only formalin-induced pain behavior was reduced (Cockayne et al., 2000; Souslova et al., 2000), further supporting the notion that P2X\(_3\) receptor activation does not play an important role in acute sensitization but may be involved in chronic pain syndromes.

The concept that ATP has sensitizing actions on sensory neurons (i.e., augments the excitability of other stimuli) is supported by a number of other studies. Tominaga et al. (2001) reported that 100 \( \mu M \) ATP augments the capsaicin-evoked and low pH-evoked currents in HEK293 cells transfected with the vanilloid receptor, VR1, and capsaicin-evoked inward currents in rat dorsal root ganglia neurons. This concentration of ATP did not evoke inward current when given alone, supporting the notion that ATP sensitizes sensory neurons. In an analogous manner, Zimmermann et al. (2002) recently reported that high micromolar concentrations of ATP and UTP did not increase iCGRP release from an in vitro preparation of the dura mater, but did enhance low pH-evoked iCGRP release. On the other hand, Molliver et al. (2002) demonstrated that in addition to prolonged increase in AMP response element-binding protein phosphorylation, ATP or UTP also evoked slow-onset and long-lasting action potential firings in nociceptive sensory neurons, and these effects were mediated by P2Y\(_2\) receptors. Another study has reported a direct increase in CRGP release after exposure to the purinergic agonist, UTP (Sanada et al., 2002). It was shown that 100 \( \mu M \) ATP or UTP increases free intracellular calcium in rat small-diameter sensory neurons grown in culture and that the same concentration of UTP increased iCGRP release. The increase in free calcium was largely from intracellular calcium stores. The inconsistency between their release data and ours may be related to the dose of UTP that was used (since we only tested 10 \( \mu M \)), the difference in dorsal root ganglia cells from embryonic or adult origin, or cell culture conditions.

In an attempt to ascertain which receptors are responsible for the sensitizing actions of ATP, we used a number of purinergic agonists and antagonists. One limitation of this

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**Fig. 6.** Bisindolylmaleimide blocks ATP-induced augmentation of capsaicin-evoked neuropeptide release from cultured rat sensory neurons. The ordinates represent the amount of iCGRP released in femtomoles per well per 10-min incubation. Data are mean ± S.E.M. values of the amount of peptide released for the number of wells indicated. Open columns represent release when cells were exposed to HEPES buffer alone or buffer with 10 \( \mu M \) ATP in the absence or presence of 100 nM bisindolylmaleimide I (Bim), whereas shaded columns indicate release when cells were exposed to 30 nM capsaicin (CAP) + drugs. An asterisk indicates the statistically significant difference between release caused by capsaicin alone and by capsaicin in the presence of 10 \( \mu M \) ATP. A cross indicates the statistically significant difference between releases caused by capsaicin in the presence of 10 \( \mu M \) ATP and capsaicin in the presence of 10 \( \mu M \) ATP and 100 nM Bim.
ATP Increases Evoked Peptide Release


References


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